PAPER ELECTROPHORETIC STUDIES ON THE RELATIONSHIP BETWEEN THE FREE AMINO ACID CONTENT AND REGENERATION IN PLANARIA

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ABSTRACT

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Paper Electrophoretic Studies on the Relationship Between the Free Amino Acid Content and Regeneration in Planaria

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A comparison of the regenerative activity and the amino acid content was made on 4 transverse sections of planaria, designated as Sections I, II, III and IV.

Transections were made of 100 animals and these were allowed to regenerate. The amount of regeneration of each section, time involved, and number of abnormal head regenerates were observed with a small stereoscopic microscope. Thirty additional animals were transected and samples from these were analyzed for amino acid content by paper electrophoresis.

Regeneration proceeded at a faster rate in Section I and there was a larger number of free amino acids in this Section. There was a much larger number of abnormal regenerates in Section III than in the other sections. This section contained only 4 amino acids. The free amino acids present in all the sections were lysine, arginine, histidine, glycine, alanine, leucine, phenylalanine, tyrosine, tryptophane, glutamic acid, aspartic acid and cystine.

Experimental data seem to suggest a relationship between regenerative activity and amino acid content in 4 transverse sections of planaria.

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CHAPTER I

INTRODUCTION

Regeneration, the restoration of lost or amputated parts, is a capacity manifested to a high degree among invertebrates and many cold-blooded vertebrates. It essentially diminishes as one phylogenetically ascends the evolutionary scale to regeneration in man where there is the replacement of hair, finger-nails, skin, and certain other cells.

Planaria have been the subject of extensive research because of their extraordinary capacities for regeneration. They are also excellent for research in that the body is clearly differentiated into anterior and posterior ends and that within this phylum, platyhelminthes, the first appearence of a central nervous system is seen.

Amino acids are the units from which protein molecules are built and these contain at least one amino group and a carboxyl group, hence the designation. Present information indicates that amino acids exist as zwitterions; that is, both groups are charged so that the net charge is zero at their isoelectric point. There is no migration toward the anode or cathode at this electrically neutral point. If however, an amino acid is dispersed in an acid solution below its isoelectric pH, the amino acid will possess a positive charge, and there is a migration toward the cathode. In alkaline solution, the amino acid will possess a negative charge and there is a migration toward the anode.

Electrophoresis is the migration of charged particles in a buffered solution between two electrical poles. It is a micro-technique based upon the principle that charged molecules migrate in an electrical field

set up across a strip of filter paper saturated with a buffer. The individual components of a mixture will migrate at different velocities in an electric field in accordance with their charge and particle size.

Since protein metabolism is known to be important in any morphogenetic process, this study was undertaken in order to determine the possibility of a relationship between the free amino acid content and regenerative ability in four different sections of planaria as determined by electrophoresis.

CHAPTER II

REVIEW OF LITERATURE

Some of the first studies on regeneration in planaria were concerned with not only the capacity for regeneration of artificially divided planaria, but also the effect of temperature on the rate of regeneration, the power of anterior regeneration, and heteromorphosis. Morgan (1904), Bardeen (1901), Lillie (1901), and others, contributed new and varied techniques in determining regenerative potencies of planaria. Lillie (1901) in his work with Planaria dendrocoelum demonstrated that although tissue will grow at any transverse level behind the region immediately back of the eyes in the form of a tail, the capacity for regeneration of a head was limited to the anterior third or fourth of the body.

Child (1911) proposed that not only was the head region dominant over the regions posterior to it, but in general a given area of the body was dominated by regions anterior to it, and also dominant over regions posterior to it. He felt that the increased metabolic rate at the anterior end was an explanation for its dominance over regions posterior to it. Child (1914), in working with Planaria dorotocephala, reported that head determination occurred during the period of stimulation (six to eight hours after transection) and that the greater the amount of stimulation to a section, the less likely a head was to develop. He advanced the theory that the essential feature of the organism was an axial physiological gradient. Although his most extensive work was done with Planaria dorotocephala, his experiments also included other species of planaria and some plants. All of this work supported his theory of the axial physiological gradient in the organism.

Other studies emphasized the role played by amino acids and protein snythesis in the phenomenon of regeneration. Hammett (1943), working with Obelia, reported that growth in this coelenterate was something more than an increase in weight or size, but rather, a combination of the activity of elements in the initiation, proliferation, differentiation, organization, and increase in substance. Hammett and Chapman (1938) observed that the amino acids in Obelia were liberated in the dissolution of the distintegrating cell mass and were presumably transferred to the rest of the colony by the hydroplasm. He further suggested that the growing animal used free amino acid for its substance increase and that the amino acids liberated during the catabolism of the senile structure may be re-utilized in the growth of the parts.

Needham (1950) demonstrated that tadpoles could be reared to metamorphosis on a diet in which a single amino acid was the sole source of nitrogen. This indicated that the amino acids differ in their effects on growth and differentiation. Arginine, cystine, lysine and phenylalanine promoted growth but very little differentiation. Tyrosine and tryptophane promoted differentiation, rather than growth, and produced small well-developed animals. Leucine and alanine suppressed both growth and differentiation; whereas, histidine and aspartic acid hardly promoted maintenance. Di-iodotyrosine has been used to hasten metamorphosis, but this would be retarded if a "growth acid" was given simultaneously.

Needham (1952) reported that the most important of any morphogenetic process was protein metabolism. He indicated that the regressive phase was characterized by hydrolysis of proteins and that the progressive phase was accompanied by protein synthesis. However, protein flow (proteolytic factor which escapes from the wound into general circulation), or suppression of

flow has not been shown to promote regeneration. He reported that protein flow was significant in that it increased the production of alkaline phosphatase essential for the repair phase. He suggested that the main function of alkaline phosphatase was the liberation of energy by breakdown of high energy phosphate, the immediate source of high energy phosphate bonds being possibly that of adenyl triphosphate (ATP). He found that ATP did promote differentiation. He also observed that the products of protein catabolism (including amino acids) probably were most concentrated between the sixteenth and twentieth days of regeneration.

According to Needham (1952), Hammett and associates, using Obelia hydranths, examined the individual effects of most amino acids on growth and differentiation. Those having promoting effects were found to be glycine, dl-alanine, l-serine, d(-)threonine, d-valine, norvaline, l-leucine, isoleucine, l-cysteine, dl-methionine, d-glutamic acid, l-aspartic acid, d-lysine, d-arginine, l-histidine, l-proline, l-hydroxyproline, dl-phenylalanine, l-phenylalanine and l-tryptophane. Needham (1952) also reported on Lecamp's work with amino acids and their effectiveness on regeneration in planaria. The order of amio acids according to their decreasing efficiency on regeneration was histidine, arginine, tryptophane, lysine, cysteine, glycine, and glutamic acid. According to Needham, Lecamp also felt that there was an optimum concentration for these important and basic materials.

Several investigators have presented evidence to support the theory that there was a definite dependence of organ regeneration upon the nervous system of both vertebrates and invertebrates. Needham reported that the nerve supply was perhaps necessary for regeneration in most animals, including arthropods, and that the nerve fibers promoted protein syntheses in

neighboring tissues. According to Reyer (1962), Lender demonstrated that a substance that could be extracted from head ganglia would promote regeneration of ocelli in planaria. He suggested that this substance perhaps caused the migration and accumulation of regenerative cells at the site of the wound.

Singer (1956), using a technique in which chemical solutions were directly introduced into regenerated tissue at a rate of a thousandths of a cubic centimeter per hour, suggested that it was possible that some substance released by the nerve promoted growth. In amphibian regeneration this substance which served to promote growth had been identified as either, acetylcholine or sympathin. Experiments have shown that regeneration of fully innervated limb stumps was inhibited if the acetyl-choline mechanism was interrupted. However, denervated regenerates would not grow in solutions of acetyl-choline or in combination with eserine.

The cellular basis of regenerative activity with respect to the nucleic acids has been the concern of many biologists. Hay (1962) conducted studies concerned with changes which take place during regeneration of the forelimb of Ambystoma larvae, with special attention given to developing cartilage and muscle. She observed that the mesenchymal cells, which are precursors of muscle and cartilaginous cells, contained prominent nucleoli and large nuclei. The presence also of numerous ribonucleoprotein granules in the cytoplasm indicated that the cells were actively engaged in nucleic acid and/or protein synthesis. In addition, observations from electron microscopic studies demonstrated that dedifferentiating cells, as they began to divide, were sources of nucleic acid and protein synthesis.

Bronsted and Bronsted (1953) described experiments in which the influence of ribonucleic acid (RNA) on regeneration in starved animals was determined.

The three planarian species used for the investigation were Dendrocoelum lacteum, Planaria vitta, and Euplanaria lugutiris. The results of these experiments indicated that RNA in concentrations of 1:10,000 accelerated regeneration in the three species of starved animals. Results also indicated that RNA served as a stimulating substance, and not as a food source.

Hoff-Jørgensen, Løvtrup and Løvtrup (1953) were interested primarily in determining whether starvation resulted in a reduction of the volume of the individual cells, or in a decrease in cell number, or both. Using the fresh water species, Polycelis nigra, they investigated the changes in total nitrogen and DNA in these starving animals. The results of the nitrogen determinations indicated a reduction of 14% of its initial value. There was also a very small, almost neglible, decrease in DNA content.

Gray (1951) stated that the phenomenon of electrical migration was observed by Alexander Reuss in 1807 when he allowed an electric current to pass through glass tubing containing water and clay. The colloidal clay particles, being negatively charged, moved toward the anode. Gray also reported that Michael Faraday in England and E. H. Dubois - Reymond in Germany further explored this phenomenon and showed that any positively charged particle will travel toward the cathode and negatively charged particles will move toward the anode. According to Gray, they also observed that there was a definite relationship between mobility and the surface charge or surface potential of the substance.

Bier (1959) reported an example of interest in electrophoretic mobilities, which depended upon the pH of the solution as shown by Michaelis in his determination of the isoelectric points of enzymes. This was done by determining the direction of the migration in a U-tube and by examining the

arms of the tube for enzymatic activity after current had passed through.

Gray (1951) stated that the moving-boundary method was contributed by Tiselius in 1938, who developed the apparatus for this type of electrophoretic analysis. He analyzed the serum of horse blood and observed that globulin was a mixture of alpha, beta, and gamma globulins.

CHAPTER III

MATERIALS AND METHODS

Planaria were secured from the Carolina Biological Supply Company, Burlington, N. C., placed in finger bowls filled with dechlorinated water and fed beef liver approximately twice a week. After three weeks of acclimation to the laboratory conditions, the animals were starved from seven to 13 days. After the period of starvation, 40 animals were transected with a sharp scalpel into four pieces and assigned to Sections I, II, III and IV. The anterior end of the animal was transected first and designated Section I. The posterior end was then cut and named Section IV. The remaining medial portion was cut into two approximately equal parts with the anterior end being labelled Section II and the posterior end as Section III. Ten pieces of each section were transferred to dechlorinated water in Stender dishes and left to regenerate. The regenerating sections were not fed, but the water was changed twice per week. The amount of regeneration for each section, the time involved, and the number of abnormal head regenerates in each section, were observed with a stereoscopic microscope. The remaining 30 planaria were used for determining the amino acid content.

Sections to be used for determining amino acid content were placed in a 75% solution of ethanol, homogenized, and centrifuged at 9,000 R.P.M. for 15 minutes. The supernatant was decanted, evaporated to dryness over a water bath, and dissolved in a small amount of 75% ethanol. The samples were then transferred to small glass tubes and refrigerated.

The buffer, with a pH of 2.0, was prepared by mixing 58 ml of glacial acetic acid with 26 ml of a 25% solution of formic acid. This mixture was diluted to two liters. Alkaline ninhydrin was prepared by adding 0.2 gm of

ninhydrin to 100 ml of ethanol and 0.5 ml of aqueous normal potassium hydroxide.

The Spinco Model R Paper Electrophoresis Cell and Spinco Duostat were used to determine the amino acid content of sections. The paper wicks were inserted into the chamber of the electrophoretic cell and eight paper strips were aligned across the rack. The support stand was then folded, locked into position, and 800 ml of the buffer solution were poured into the chamber. The rack and paper strips were then placed on the support stand. The cover was placed on the cell, 200 ml of the buffer were poured over the strips, and the cell was tilted to equalize the liquid in both compartments. At the end of 15 minutes, a sample of the mixture to be analyzed, (0.006 ml), was applied to the surface of the paper strips with a Spinco applicator. The cell was sealed with tape, covered, and connected to the Duostat. A potential of 150 volts was introduced for 16 hours. At the end of 16 hours, the strips were removed and placed in an oven at 110C for 15 minutes to dry. Papers were then dipped rapidly through alkaline ninhydrin and, after evaporation had occurred, were again placed in the oven and heated at 110C until maximum color was obtained. A few drops of glacial acetic acid and a few drops of pyridine were added to ninhydrin before the strips were dipped.

Samples from 14 known amino acids were run on individual strips. These amino acids were lysine, arginine, histidine, glycine, alanine, leucine, phenylalanine, tyrosine, tryptophane, glutamic acid, aspartic acid, cystine, cysteine acid and serine. Strips with samples from Section I, II, III and IV were compared with the known strips in order to determine the amino acids present in each section.

CHAPTER IV

EXPERIMENTAL RESULTS

Regenerative activity of four transverse sections of planaria was compared to the amino acid content of these sections. Regeneration of sections involving a total of 100 planaria was begun on May fifteenth and concluded on July tenth. In each experiment ten planaria were transected and placed in Stender dishes to regenerate. Experimental conditions were kept as much alike as possible for all sections. Regenerates were kept in a partially opened laboratory drawer and the temperature ranged between 20-25 centigrade. All planaria were starved for seven days and no food was given during the period of regeneration.

The time required for regeneration extended from a period of six-to-nine days for Section I, seven-to-nine days for Section II, eight-to-11 days for Section III, and eight-to-ten days for Section IV. The average number of days for regeneration for Section I was seven, eight, for Section II, ten for Section III, and nine for Section IV. It was observed that the time for regeneration of all the sections decreased during the last regenerative periods. It may be that the rise in temperature was perhaps responsible for the increased rate in regeneration. It was also observed that regeneration proceeded at a faster rate in Section I, and at the slowest rate in Section IV. The exact time at which regeneration was complete is difficult to determine, but the process was considered complete when a clear, well defined, anterior or posterior portion could be observed. The regenerated tissue was clearer and rather easy to distinguish; however, the numbers assigned for regeneration rates must be approximate ones.

From a total of 100 sections made at ten different periods, there were 95 regenerates in Section I, 97 in Section II, 85 in Section III, and 96 regenerates in Section IV. The largest number of regenerates was obtained in Section II, and the smallest number in Section III.

Abnormal regenerates were considered to be any malformation of head or eye structures. A normal head is shown in Figure 1, an abnormal head (teratomorphic) in Figure 2, abnormally large eyes in Figure 3, eyeless (anopthalmic) in Figure 4, and approximating eyes in Figure 5. There were two abnormal head regenerates in Section II, 64 in Section III and 5 in Section IV.

Electrophoretic analyses of samples from the 4 sections revealed the presence of the following free amino acids: Lysine, arginine, histidine, glycine, alanine, leucine, phenylalanine, tyrosine, tryptophane, glutamic acid, aspartic acid and cystine. Section I (Figure 6) contained lysine, arginine, histidine, glycine, alanine, leucine, phenylalanine and tryptophane. Tyrosine, tryptophane, glutamic acid, and cystine were found in Section II (Figure 6). Samples of Section III (Figure 6) revealed the presence of alanine, leucine, tyrosine, and cystine, and observation of Section IV (Figure 6) indicated the presence of leucine, tryptophane, glutamic acid, aspartic acid and cystine.

In general, the spots representing the free amino acids were not intensely colored, suggesting that the concentrations were low. Cystine (Section III) and tryptophane (Section IV) were most intensely colored.

CHAPTER V

DISCUSSION

The results of these experiments on regeneration seem to support the axial gradient theory proposed by Child (1911). This theory states that not only is the head region dominant over the regions posterior to it, but a given area of the body is dominated by regions anterior to it, and is dominant over regions posterior to it. Regeneration not only proceeded at a faster rate in Section I, but this section was found to contain the largest number of free amino acids. This also seems to agree with Needham (1952) in that the large number of free amino acids perhaps resulted from extensive protein break-down which is characteristic of the regressive phase of regeneration. Analysis revealed only five free amino acids in Section IV and this is perhaps indicative of reduced metabolic processes.

Needham (1950) listed arginine, cystine, lysine, and phenylalanine as promoting growth but very little differentiation. It is possible that the large concentration of cystine found in Section III was a contributing factor to the large number of abnormal heads in that section of regenerates. He also listed tyrosine and tryptophane as influencing differentiation rather than growth. This may be an explanation for the observation that the smallest number of abnormal heads in Section II can be attributed to the presence of tyrosine and tryptophane.

Needham (1952) reported that Lecamp, in testing the effectiveness of amino acids on regeneration in planaria, found that histidine, arginine, tryptophane, lysine, cystine, glycine and glutamic acid were effective on regeneration in planaria. The strips of Section I revealed that, of the

above group, lysine, arginine, histidine, tryptophane and glycine were present. Tryptophane, glutamic acid and cystine were present in Section II, cystine in Section III, and tryptophane, glutamic acid and cystine in Section IV. There were, however, other amino acids found during the analyses of these sections that were not included on Lecamp's list. They were alanine, leucine, phenylalanine, and aspartic acid.

Observations revealed that some of the abnormal regenerates were devoured by the normal ones. Consequently, it is possible that the actual number of regenerates in Section III was larger than the number quoted. This also influenced the number of abnormal regenerates.

CHAPTER VI

SUMMARY AND CONCLUSIONS

1. Planaria were transected into 4 pieces and assigned to Sections I, II, III and IV. The anterior piece of the animal was designated Section I, the posterior piece, Section IV. The anterior end of the medial portion was Section II and the posterior end, Section III.

2. Increased metabolism in Section I was perhaps responsible for its faster rate of regeneration.

3. The large number of amino acids in Section I may also result from increased metabolism.

4. The presence of a very large number of abnormal heads in Section III may be due to decreased metabolism, the lack of essential amino acids, the large concentration of cystine, or a combination of these factors.

5. This study indicates that there may be some relationship between free amino acid content and regenerative activity of 4 transverse sections of planaria.

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Figure 1

1. Normal head of regenerate from Section III.

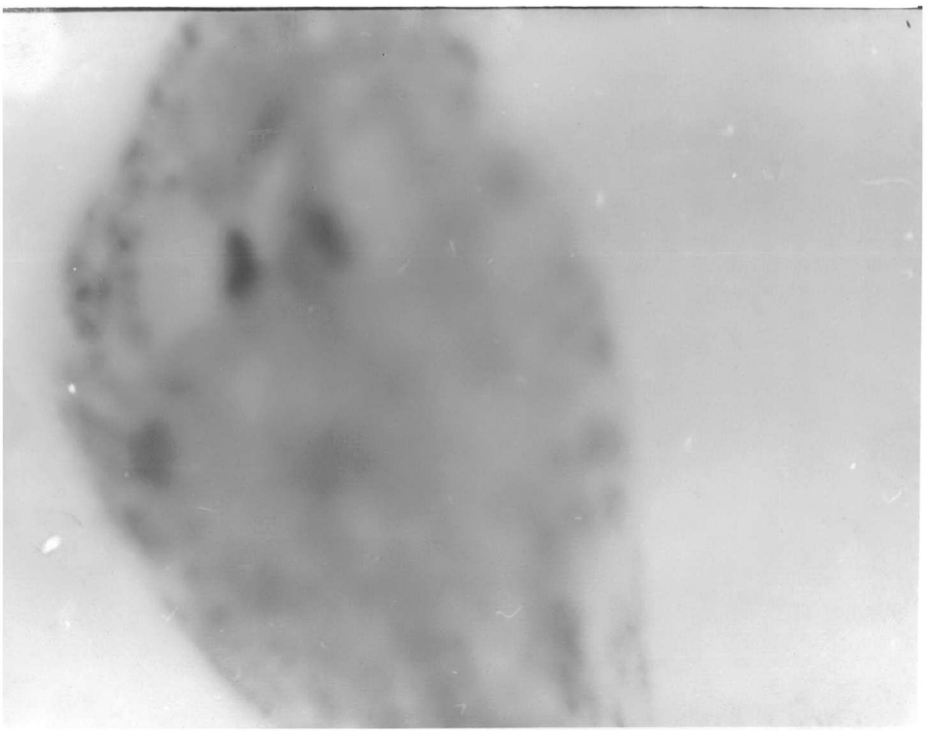

Figure 2

2. Head of regenerate from Section III showing teratomorphic condition.

Figure 3

3. Head of regenerate from Section III showing abnormally large eyes.

Figure 4

4. Head of regenerate from Section III showing anopthalmic condition.

Figure 5

5. Head of regenerate from Section III showing approximating eyes.

Figure 6

6. Paper strips showing free-amino acids of Sections, I, II, III and IV
(1) Lysince (2) Arginine (3) Histidine (4) Glycine (5) Alanine (6) Leucine
(7) Phenylalanine (8) Tyrosine (9) Tryptophane (10) Glutamic Acid
(11) Aspartic Acid (12) Cystine

I
II
III
IV
1
2
3
4
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12
13